PATENT
Atty. Docket No. 2303.2B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS: David H. Gelfand et al.

SERIAL NO.: To be Assigned GROUP ART UNIT:

FILED: Herewith EXAMINER:

DOCKET NO.: 2303.2B

TITLE: STABILIZED THERMOSTABLE ENZYME COMPOSITIONS

DECLARATION UNDER 37 C.F.R. § 1.132

Hon. Commissioner of Patents
and Trademarks
Washington, D. C. 20231

Sir:

I, James Akers do hereby declare as follows:

1. I am employed by Roche Molecular Systems as a Senior Scientist, and have been so employed since the merger of Cetus and Roche to establish Roche Molecular Systems in December of 1991. Prior to my employment with Roche Molecular Systems, I was employed by Cetus Corporation for about 6 years; about 3 years in reagent development and about 3 years in instrument development. I was also employed as a chief technologist by various hospital and clinical reference laboratories prior to my employment by Cetus. Overall, I have about 20 years of laboratory experience.

2. In relation to the prosecution of the present patent application, I have performed three (3) experiments which are described below.

Experiment No. 1:

Reverse Transcriptase Titration Experiment

This experiment was conducted to determine whether a reverse transcriptase enzyme (RT) maintains the same functional activity if the non-ionic polymeric detergent, NP-40, is removed from the enzyme's buffer. The standard preparation of RT contains 0.01% NP-40. The functionality of the two RT preparations was compared by testing in the standard RNA-PCR protocol described in the package insert of the Roche RNA-PCR kit.

Briefly, the RNA-PCR protocol involves the conversion of genomic RNA to cDNA by addition of reverse transcriptase, a DNA primer and deoxynucleoside triphosphates to the genomic RNA. Then, a PCR reaction is run in a thermocycler with the cDNA, a thermostable DNA polymerase, deoxynucleoside triphosphates and selected primers for the desired DNA sequence to be amplified.

The standard RT storage buffer (designated Buffer #1) contained:

20 mM Tris
100 mM NaCl
1 mM DTT
0.01% NP-40
0.1 mM EDTA
50% (v/v) glycerol
pH 7.5.

The experimental RT storage buffer without non-ionic polymeric detergent (designated Buffer #2) contained:

20 mM Tris
100 mM NaCl
1 mM DTT
0.1 mM EDTA
50% (v/v) glycerol
pH 7.5.

RT dilutions in Buffer #1 or in Buffer #2 were used at concentrations of 50, 25, 12.5, 6.25 or 3.125 U/µl storage buffer in the standard RNA-PCR protocol. In addition, a negative control using the RNA-PCR kit RT at 50 U/λ with no RNA added, and a positive control using the RNA-PCR kit RT at 50 U/λ were also run.

The standard RNA-PCR assay was then run with random hexamers and carrier RNA in order to stress the RT for each RT preparation. The post-PCR reactions were run on 4% TBE agarose gel (1% SeaKem, 3% NuSieve) with 15λ of 10 mg/ml ethidium bromide/200 ml of 4% agarose gel. The electrophoresis was conducted for approximately 2 hours at 175 volts in 1X TBE Buffer.

The results of this experiment showed the following:

(1) The negative controls were negative.
(2) The positive controls were positive.
(3) Even with 1 µg of carrier RNA and using random hexamers, equivalent functionality was present between 50 U of RT/reaction and 6.25 U of RT/reaction. The 3.125 U of RT/reaction was slightly less intense.

(4) The functionality of RT appears to be equivalent whether the RT is diluted in Buffer #1 (normal RT storage buffer with non-ionic detergent, NP-40) or in Buffer #2 (storage buffer without NP-40).

Experiment No. 2:

AmpliTaq DNA Polymerase Titration Experiment

This experiment was conducted to determine whether a Taq DNA polymerase enzyme maintains the same functional activity if the non-ionic polymeric detergents, NP-40 and Tween 20, are removed from the enzyme's buffer. The standard preparation of Taq DNA polymerase contains 0.5% NP-40 and 0.5% Tween 20. The functionality of the two Taq DNA polymerase preparations was compared by testing in the standard RNA-PCR protocol.

The standard Taq DNA polymerase storage buffer (designated Buffer #1) contained:

20 mM Tris
100 mM KCl
1 mM DTT
0.5% NP-40
0.5% mM Tween 20
0.1 mM EDTA
50% (v/v) glycerol
pH 8.0.

The experimental Taq DNA polymerase storage buffer without non-ionic polymeric detergents (designated Buffer #2) contained:

20 mM Tris
100 mM KCl
1 mM DTT
0.1 mM EDTA
50% (v/v) glycerol
pH 8.0.

Taq DNA polymerase dilutions in Buffer #1 or in Buffer #2 were used at concentrations of 5, 4, 3, 2 or 1 U/λ storage buffer in the RNA-PCR protocol. In addition, a negative control using the RNA-PCR kit Taq DNA polymerase at 5 U/λ with no RNA added, and a positive control using the RNA-PCR kit Taq DNA polymerase at 5 U/λ were also run.

The standard RNA-PCR assay was then run with carrier RNA to simulate genomic RNA load for each Taq DNA polymerase preparation. The post-PCR reactions were run on 4% TBE agarose gel (1% SeaKem, 3% NuSieve) with 15λ of 10 mg/ml ethidium bromide/200 ml of 4% agarose gel. The electrophoresis was conducted for about 2 hours at 175 volts in 1X TBE Buffer.

The results of this experiment showed the following:

(1) The negative controls were negative.
(2) The positive controls were positive.
(3) The Taq DNA polymerase diluted in Buffer #1 (normal Taq DNA polymerase storage buffer with the non-ionic polymeric detergents, NP-40 and Tween 20) produced clearly visible bands at all dilutions, even 1 U/λ.

(4) No bands were visible for the Taq DNA polymerase preparations in Buffer #2 (storage buffer without NP-40 or Tween 20), thus indicating that there is a minimum amount of non-ionic polymeric detergent necessary for the functional activity of Taq DNA polymerase, and the amount of non-ionic polymeric detergent necessary is greater than the 0.001% final concentration of NP-40 carried over by the RT used in the first steps of the protocol.

Experiment No. 3:

Taq DNA Polymerase Titration; Detergent v. Gelatin

This experiment was conducted to determine whether Taq DNA polymerase maintains the same functional activity if the non-ionic polymeric detergents, NP-40 and Tween 20, are removed from the enzyme's storage buffer and replaced by gelatin. The standard preparation of Taq DNA polymerase contains 0.5% NP-40 and 0.5% Tween 20. In the experimental Taq DNA polymerase preparation, these two non-ionic polymeric detergents were replaced by 250 µg/ml of gelatin. The functionality of the two Taq DNA polymerase preparations was compared by testing in the RNA-PCR protocol.

The standard Taq DNA polymerase storage buffer (designated Buffer #1) contained:

20 mM Tris
100 mM KCl
1 mM DTT
0.5% NP-40
0.5% Tween 20
0.1 mM EDTA

50% (v/v) glycerol
pH 8.0.

The experimental Taq DNA polymerase storage buffer with non-ionic polymeric detergents replaced by gelatin (designated Buffer #2) contained:

20 mM Tris
100 mM KCl
1 mM DTT
250 µg/ml gelatin
0.1 mM EDTA
50% (v/v) glycerol
pH 8.0.

Taq DNA polymerase dilutions in Buffer #1 or in Buffer #2 were used at concentrations of 5, 4, 3, 2 or 1 U/λ storage buffer in the RNA-PCR protocol. In addition, a negative control using the RNA-PCR kit Taq DNA Polymerase at 5 U/λ with no RNA added, and a positive control using the RNA-PCR kit Taq DNA polymerase at 5 U/λ were also run.

The standard RNA-PCR assay was then run with carrier RNA to simulate genomic RNA load for each Taq DNA polymerase preparation. The post-PCR reactions were run on 4% TBE agarose gel as described in Experiment No. 2. The electrophoresis was also conducted as described in Experiment No. 2.

The results of this experiment were as follows:

(1) The negative controls were negative.
(2) The positive controls were positive.
(3) The Taq DNA polymerase preparations diluted in Buffer #1 (standard storage buffer with the

non-ionic polymeric detergents NP-40 and Tween 20) produced visible bands at all dilutions.

(4) The Taq DNA polymerase preparations diluted in Buffer #2 (storage buffer with the non-ionic polymeric detergents replaced by gelatin) did not produce bands at any dilution, thus indicating that some amount of detergent is necessary for Taq DNA polymerase functionality, and that amount is greater than the 0.001% NP-40 carried over by the RT used in the first steps of the protocol. Furthermore, gelatin does not restore the functionality of Taq DNA polymerase in the absence of non-ionic polymeric detergent.

3. Based on the results of Experiment Nos. 1 and 2, described above, it is my conclusion that the effects of a non-ionic polymeric detergent on an RT enzyme is not predictive of the effects of a non-ionic polymeric detergent on a thermostable nucleic acid polymerase.

4. Based on the results of Experiment No. 3, described above, it is my conclusion that gelatin is not equivalent to a non-ionic polymeric detergent for maintaining the functionality of a thermostable nucleic acid polymerase.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States

Code, and that such willful false statements may jeopardize the validity of the present application or any patent issuing hereon.

James Akers

4-22-92

Date

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PATENT
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DECLARATION UNDER 37 C.F.R. §1.132

Hon. Commissioner of Patents
and Trademarks
Washington, D. C. 20231

Sir:

I, David H. Gelfand do hereby declare as follows:

1. I am a co-inventor of the subject matter claimed in the present application.

2. I am familiar with the Office Actions which were mailed by the Examiner on September 4, 1990 and on May 3, 1991. I am also familiar with the references cited by the Examiner in those Office Actions.

3. In the Office Action mailed May 3, 1991, the Examiner was skeptical of our assertion that the Goff et al. reference which relates to reverse transcriptase enzyme does not suggest stability problems associated with thermostable polymerases (see Office Action of May 3, 1991, page 5). In fact, on the same page of the Office Action, the Examiner stated that:

> There is seen nothing to lead one to believe that a polymerase being thermostable changes its characteristics so as to make unexpected a nonionic detergent functioning to stabilize the enzyme as suggested by Goff et al. and, if needed, Fuller et al. or Spiegelman.

4. Based on the respective amino acid sequences of Maloney murine leukemia virus reverse transcriptase (MuLV RT) (the reverse transcriptase described by Goff et al.) and Thermus aquaticus (Taq) DNA polymerase, there is no reasonable basis for asserting a relatedness of these two enzymes. I ran the amino acid sequences for both of these enzymes through a computer program which looks for amino acid similarities, and none were found between MuLV RT and Taq DNA polymerase. In fact, the degree of relatedness of MuLV RT and Taq DNA polymerase was slightly worse than the average degree of relatedness from 10 computer runs of the same amino acids which had been randomized. Specifically, the "quality of best alignment" for MuLV RT and Taq DNA polymerase was 220, and the average "quality of best alignment" for the randomized amino acids was 223. Also, the significant difference between the isoelectric points of these two enzymes is further evidence of the unrelatedness of MuLV RT and Taq DNA polymerase. The isoelectric point of MuLV RT is 8.8, and the isoelectric point of Taq DNA polymerase is 6.3.

5. I also ran the same computer program to compare the similarities of the amino acid sequence of MuLV RT and the amino acid sequences of bovine serum albumin, E. coli β-galactosidase and glycogen phosphorylase B. The computer program showed that MuLV RT is also not related to any of these proteins.

6. In addition, I ran the same computer program to compare the similarities of the amino acid sequences of Taq DNA polymerase and E. coli DNA polymerase I. The program shows a reasonable sequence similarity between these two enzymes. In

contrast to the comparison of MuLV RT and Taq DNA polymerase (paragraph 4, above), the "quality of best alignment" for Taq DNA polymerase and E. coli DNA polymerase I was 609, while the average "quality of best alignment" of 10 randomized series of the same amino acids was 277± 5.7.

7. Based on these findings of the unrelatedness of MuLV RT and Taq DNA polymerase, it is my opinion that the properties and behavior of MuLV RT are not predictive of the properties and behavior of a thermostable nucleic acid polymerase.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the present application or any patent issuing hereon.

David H. Gelfand

4-21-92
Date

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Hon. Commissioner of Patents
and Trademarks
Washington, D. C. 20231

Sir:

We, David H. Gelfand, Susanne Stoffel and Randy Saiki are co-inventors of the claimed subject matter of the above-captioned application. We are familiar with the Office Actions mailed September 4, 1990 and May 3, 1991, in which the Examiner rejected the claims of this application over the MBR product information sheet of June 8, 1987. Prior to May 1, 1987, we reduced the claimed invention to practice in the United States. Evidence of our reduction to practice is shown by the attached letter and accompanying enclosure.

The letter, addressed to Dr. James F. Wick of MBR, shows that, prior to May 1, 1987, David H. Gelfand sent to MBR a detailed description of our Taq polymerase purification protocol, including a description of a stabilized enzyme composition of the present claims. The protocol attached to the letter clearly shows that the stable enzyme compositions of the present invention were reduced to practice and shown to MBR well before the date of the product information sheet.

The Examiner's attention is drawn to page one of the protocol, under "Polymerase Assay," where, at point six, the composition of the enzyme diluent is given. The diluent contains non-ionic polymeric detergent. In addition, at the very last page of the protocol, the Examiner's attention is also directed to the composition of 2X storage buffer, shown in the last four lines of the last page, in which the thermostable nucleic acid polymerase is stored. This storage buffer also contains non-ionic polymeric detergent.

We believe that the attached letter and protocol clearly show that we reduced the claimed invention to practice well before the date of the MBR product sheet, and we further believe that MBR derived the information on the product information sheet from the material accompanying this declaration, which were sent to MBR before May 1, 1987, and well before the date of the MBR product information sheet.

We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing hereon.

David H. Gelfand — 4-21-92
Date

Susanne Stoffel — 4-21-92
Date

Randy Saiki — 4-21-92
Date

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